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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF
HUANG ET AL.
APPLICATION NO: 10/648,593
FILED: AUGUST 26, 2003
FOR: METHODS OF USING EphA2 FOR PREDICTING ACTIVITY OF COMPOUNDS THAT INTERACT WITH AND/OR MODULATE PROTEIN TYROSINE KINASES AND/OR PROTEIN TYROSINE KINASE PATHWAYS IN BREAST CELLS

Art Unit: 1652
Examiner: SWOPE, SHERIDAN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Sir:

1. I, Fei Huang, Ph.D., am an applicant of the patent application Serial No. 10/648,593 identified above and co-inventor of the subject matter described and claimed in this patent application.

2. A true and accurate copy of my *curriculum vitae*, which evidences my expertise and credentials in the field of Pharmacogenomics and Biochemistry, is attached herewith and labeled **Exhibit A**.

3. I have performed an experiment, or directed or caused an experiment to be performed, to demonstrate that the 137 predictor polynucleotides listed in Table 2 of the instant application, including the EphA2 receptor, caveolin 1, and caveolin 2

polynucleotides, in addition to the 40, 15, and 7 predictor polynucleotide sets listed in Tables 2, 4, and 5 of the instant application, respectively, can predict the sensitivity and resistance of breast cancer cells with reasonable accuracy to several compound inhibitors of members of the Src family of tyrosine kinases, including e.g., Src, Fgr, Fyn, Yes, Blk, Hck, Lck and Lyn, as well as other protein tyrosine kinases, including, Bcr-abl, PDGFR, c-kit and Eph receptors. Each of these compounds are disclosed in WO 00/62778, published October 26, 2000 and are referred to herein as BMS-A, BMS-B, BMS-C, BMS-D, and BMS-E. The identity of each of these compounds is provided in Exhibit L.

The sensitivity classification of compounds BMS-A to –E, the performance of the polynucleotide expression and marker polynucleotide identification analyses in each of the 23 breast cancer cell lines are described below.

IC_{50} determination--*in vitro* cytotoxicity assay

Compounds BMS-A to -E were tested for cytotoxicity *in vitro* against a panel of twenty-three human breast cell lines available from the American Type Culture Collection, ATCC, except H3396, which was obtained from Pacific Northwest Research Institute, Seattle WA. The MCF7/Her2 cell line was established by stable transfection of MCF7 cells with the HER2 gene. Cytotoxicity was assessed in cells by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium, inner salt) assay (T.L. Riss et al., 1992, *Mol. Biol. Cell*, 3 (Suppl.):184a).

To carry out the assays, the breast cells were plated at 4,000 cells/well in 96 well microtiter plates, and 24 hours later, serially diluted drugs were added. The concentration range for the protein tyrosine kinase inhibitor compounds BMS-A to -E used in the cytotoxicity assay was from 5 μg/ml to 0.0016 μg/ml (roughly 10 μM to 0.0032 μM).

The cells were incubated at 37°C for 72 hours at which time the tetrazolium dye, MTS (333 μg/ml final concentration), in combination with the electron coupling agent phenazine methosulfate (25 μM final concentration), was added. A dehydrogenase enzyme in live cells reduces the MTS to a form that absorbs light and can be quantified spectrophotometrically at 492 nM. The greater the absorbency the greater the number of live cells. The results are expressed as an IC_{50}, which is the drug concentration required to inhibit cell proliferation (i.e. absorbance at 492 nM) to 50% of that of untreated control cells. The mean IC_{50} from multiple tests for each cell line were calculated and are provided in the

column entitled, "IC50 to X" in **Exhibits B**, **C**, **D**, **E**, and **F**, wherein "X" refers to either BMS-A, BMS-B, BMS-C, BMS-D, or BMS-E, respectively.

Resistant/sensitivity Classification

The IC_{50} of compounds BMS-A to –E for each cell line was log-transformed to $\log_{10}(IC_{50})$, and the mean $\log_{10}(IC_{50})$ across the 23 human breast cell lines was calculated. The resistance/sensitivity classification of the cell lines was classified as follows: the cell lines with $\log_{10}(IC_{50})$ below the mean $\log_{10}(IC_{50})$ of all 23 cell lines were defined as sensitive to the compound, while those with $\log_{10}(IC_{50})$ above the mean $\log_{10}(IC_{50})$ were considered to be resistant to the compound. The resistant/sensitive classification for each breast cancer cell line for compounds BMS-A to –E, as determined using IC50 calculations for each compound, are provided under the column entitled, "Sensitivity Classification based on IC50" in each of **Exhibits B**, **C**, **D**, **E**, and **F**.

The expression profile for each cell line, either in the presence or absence of compound BMS-A was determined as follows:

Polynucleotide Expression Profiling

The breast cells were grown under standard cell culture conditions: RPMI 1640 supplemented to contain 10% fetal bovine serum, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 10 mM Hepes (all from GibcoBRL, Rockville, MD). RNA was isolated from the cultured cells, either treated or untreated with compounds BMS-A to –E at 50-70% confluence using the RNeasy™ kits commercially available from Qiagen, Valencia, CA. The quality of the RNA was assessed by measuring the 28s:18s ribosomal RNA ratio using an Agilent 2100 bioanalyzer (Agilent Technologies, Rockville, MD). The concentration of total RNA was determined spectrophotometrically. 10 µg of total RNA from each cell line was used to prepare biotinylated probe according to the Affymetrix Genechip® Expression Analysis Technical Manual, 2001. Targets were hybridized to Affymetrix high density oligonucleotide array human HG-U133 set chips (Affymetrix, Santa Clara, CA). The arrays were then washed and stained using the GeneChip® Fluidics station according to the manufacture's instructions (Affymetrix Genechip® Technical Manual, 2001). The HG-U133 set contains 2 Genechip® arrays, which contain

approximately 45,000 probe sets representing more than 39,000 transcripts derived from approximately 33,000 well-substantiated human polynucleotides.

Identification Of Polynucleotides Modulated By Drug Treatment

To identify polynucleotides regulated by a protein tyrosine kinase inhibitor compound, e.g., BMS-A, a subset of 11 breast cell lines (indicated in bold in Table 1 of the instant application) having an IC_{50} ranging from 0.0055 μM to 9.5 μM were used. Cells were treated with or without BMS-A (0.4 μM) in 0.1% DMSO for 24 hours. Expression profiling was performed, and the data were analyzed using GeneChip® Expression Analysis software MAS 5.0 as described below (Affymetrix, Santa Clara, CA). The polynucleotide expression of a cell line treated with drug was compared pair-wisely to the polynucleotide expression of the same cell line without drug treatment. A change in p-value was calculated, indicating an increase, decrease or no change in polynucleotide expression. When the p-value was less than 0.0025, the change was considered to be significant. Analysis was performed for all 11 cell lines to compare the polynucleotide expression with and without drug treatment.

In addition, a pair-wise t-test with permutation analysis was applied. Polynucleotides that were significantly modulated by the drug treatment in sensitive cell lines and/or in resistant cell lines were identified. Polynucleotides whose expression was significantly changed in at least 3 cell lines were considered to be modulated by the drug.

Identifying Polynucleotides that Significantly Correlated with Drug Resistance/Sensitivity Classification

Three different statistical analyses: (i) K-mean Nearest Neighborhood algorithm with permutation test described by T.R. Golub et al., 1999, Science, 286:531-537; (ii) Pearson correlation between gene expression level and IC50; (iii) t-test to compare genes expression level of sensitive cell lines to resistant cell lines) were used to identify 137 polynucleotides that significantly correlated with drug resistance/sensitivity classification of the 23 breast cancer cell lines to compound BMS-A. Each polynucleotide was determined as being highly expressed in either sensitive or resistant breast cancer cell lines. The resulting classifications are provided in the column entitled, "Highly Expressed In" in Table

2 of the instant application and have not been reproduced herein in the interest of avoiding redundancy.

The predictive power of genes within the 137 predictor polynucleotides provided in Table 2 were used as a basis for predicting whether the 23 breast cancer cell lines were sensitive or resistant to compounds BMS-A to –E in a 'leave one out' cross validation test using a Weighted Voting algorithm as described in T.R. Golub et al., 1999, *Science*, 286:531-537. The predictive accuracies were assessed by comparing the resistant/sensitive class prediction made by the predictor gene sets based on expression pattern to the resistant/sensitive classification made by IC50 measurement for each of the 23 breast cancer cell lines.

Predictor polynucleotide sets used in the prediction consisted of one of the following: (i) the entire 137 predictor polynucleotide set; (ii) 40 predictor polynucleotide set as listed in Table 2 of the instant application; (iii) 15 predictor polynucleotide set as listed in Table 4 of the instant application; (iv) 7 predictor polynucleotide set as listed in Table 5 of the instant application; (v) the EphA2 polynucleotide alone; (vi) the EphA2 polynucleotide and the caveolin-1 polynucleotide; or (vii) the EphA2 polynucleotide and the caveolin-2 polynucleotide.

In those instances where the expression pattern was correlative to a resistant expression profile, the cell line was considered to be resistant to the tested compound. In those instances where the expression pattern was correlative to a sensitive expression profile, the cell line was considered to be sensitive to the tested compound.

The prediction strength score (referred to as "PS score" herein) of each cell line tested relative to each predictor polynucleotide set measured was calculated. The PS score refers to prediction strength for each prediction made on a cell line by the predictor set. The PS score ranges from 0 to 1, i.e., corresponding from low to high confidence in making the prediction.

Using the IC50 sensitivity/resistant results of each cell line for each compound as the standard sensitivity/resistant classification, the accuracy of either the 137, 40, 15, or 7 predictor polynucleotide sets to predict whether the breast cancer cell lines were sensitive

or resistant to compounds BMS-A to –E were calculated. The accuracy of the prediction was calculated using the following equation:

$$[(23\text{-}n) / 23] \times 100\%$$

where "n" equals the number of incorrect resistant and sensitive cell line predictions.

The results of these experiments are presented in **Exhibits B, C, D, E, and F**. **Exhibits B, C, D, E, and F,** and show the resistant/sensitivity classifications for each of the 23 breast cancer cell lines based upon the measured IC50 values for each compound BMS-A to –E, respectively, compared to the predicted resistant/sensitivity classifications as determined using either the 137, 40, 15, or 7 predictor polynucleotide set (see column "Predicted Class" for each predictor set), in addition to the calculated prediction score (see column "PS score" for each predictor set), notation of which cell line the predictor set aberrantly assigned (see cell lines marked with an asterisk (*) in the column entitled "Class Error"), and the calculated error between the two classification methods (see row entitled, "Predicted Accuracy").

4. The following comments relate to **Exhibits B, C, D, E, and F**. As shown, the 137, 40, 15, and 7 predictor polynucleotide sets predicted the resistant / sensitivity classifications of the 23 breast cancer cell lines for five of the Src tyrosine kinase / Bcr-abl, PDGFR, c-kit and Eph protein tyrosine kinase inhibitors disclosed in WO 00/62778, published October 26, 2000, with an accuracy ranging between 74% to 96%. These results unequivocally establish the utility of these predictor polynucleotides, in the identification of breast cancer cells that are resistant or sensitive to Src tyrosine kinase family members / Bcr-abl, PDGFR, c-kit and Eph protein tyrosine kinase inhibitors as demonstrated using a representative number of five such inhibitors. The results also unequivocally establish the utility of using the EphA2 receptor, caveolin 1, and/or caveolin 2 polynucleotides as predictors, either alone or in combination.

Accordingly, the utility of these predictor polynucleotide sets, in addition to the EphA2 receptor, caveolin 1, and/or caveolin 2 polynucleotides, is not solely limited to predicting the resistance or sensitivity of breast cancer cells to BMS-A, but rather to any

Src tyrosine kinase family member / Bcr-abl, PDGFR, c-kit and Eph protein tyrosine kinase inhibitor as demonstrated herein using a representative number of five such inhibitors.

5. I have performed an experiment, or directed or caused an experiment to be performed, to demonstrate that the EphA2 receptor, either alone or conjunction with either caveolin 1 or caveolin 2, can predict the sensitivity and resistance of breast cancer cells to several inhibitors of members of the Src family of tyrosine kinases / Bcr-abl, PDGFR, c-kit and Eph protein tyrosine kinases, including BMS-A, BMS-B, BMS-C, BMS-D, and BMS-E. The same IC50 calculations of the 23 breast cancer cell lines, resistant / sensitive cell line classifications, and resistant / sensitive polynucleotide expression classifications were described herein. Predictions were calculated, errors were noted, and the prediction accuracy was calculated as described herein.

The results of these experiments are presented in **Exhibits G**, **H**, **I**, **J**, and **K** and show the resistant/sensitivity classifications for each of the 23 breast cancer cell lines based upon the calculated IC50 values for each compound BMS-A to –E, respectively, compared to the predicted resistant/sensitivity classifications as determined using the EphA2 receptor polynucleotide, either alone or conjunction with either the caveolin 1 or caveolin 2 polynucleotide (see column "Predicted Class"), in addition to the calculated prediction score (see column "PS score"), notation of which cell line the predictor set aberrantly assigned (see cell lines marked with an asterisk (*) in the column entitled "Class Error"), and the calculated error between the two classification methods (see row entitled, "predicted accuracy").

6. The following comments relate to **Exhibits G**, **H**, **I**, **J**, and **K**. As shown, the EphA2 receptor, either alone or in combination with caveolin-1 or caveolin-2 accurately predicted the resistant / sensitivity classifications of the 23 breast cancer cell lines for five of the Src tyrosine kinase family / Bcr-abl, PDGFR, c-kit and Eph protein tyrosine kinase inhibitors disclosed in WO 00/62778, published October 26, 2000, with an accuracy ranging between 78% to 91% for EphA2 alone, between 61% to 74% for the combination of EphA2 and caveolin-1, and between 65% to 74% for the combination of EphA2 and caveolin-2 for each of the compounds. The results compare favorably to the 7, 15, 40, and 137 predictor polynucleotide set predictions described herein. These results unequivocally establish the

utility of the EphA2 receptor polynucleotide, either alone or in combination with either caveolin 1 or caveolin 2 polynucleotides, in the identification of breast cancer cells that are resistant or sensitive to Src tyrosine kinase family members / Bcr-abl, PDGFR, c-kit and Eph protein tyrosine kinase inhibitors as demonstrated using a representative number of five such inhibitors.

Accordingly, the utility of the EphA2 receptor, either alone or in combination with either caveolin 1 or caveolin 2 polynucleotides is not solely limited to predicting the resistance or sensitivity of breast cancer cells to BMS-A, but rather to any Src tyrosine kinase family member / Bcr-abl, PDGFR, c-kit and Eph protein tyrosine kinase inhibitor as demonstrated herein using a representative number of such inhibitors.

7. The structure of each of the Src tyrosine kinase / Bcr-abl, PDGFR, c-kit and Eph protein tyrosine kinase inhibitors used in the experiments described herein, in addition to their identity according to the BMS-X nomenclature used herein, or as used in the instant application in the case of compound BMS-A, are provided in **Exhibit L**.

8. The following comments relate to **Exhibit L**. Compound BMS-A is a compound known as Dasatinib or Sprycel®, and is marketed by the assignee of the instant application, Bristol-Myers Squibb Company, for treatment of adults in all phases of chronic myeloid leukemia (CML) (chronic, accelerated, or myeloid or lymphoid blast phase) with resistance or intolerance to prior therapy, including Gleevec® (imatinib mesylate), in addition to treatment of adults with Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) with resistance or intolerance to prior therapy. Compound BMS-A is a Src tyrosine kinase family member / Bcr-abl, PDGFR, c-kit and Eph protein tyrosine kinase inhibitor and was used in identifying the predictor polynucleotides of the instant application. Compound BMS-A was disclosed in WO 00/62778, published October 26, 2000, and is exemplified in Example 455. Compounds BMS-B, BMS-C, BMS-D, and BMS-E are additional Src tyrosine kinase family member / Bcr-abl, PDGFR, c-kit and Eph protein tyrosine kinase inhibitors and are disclosed in WO 00/62778, published October 26, 2000. Compounds BMS-B, -C, -D, and -E are exemplified in Examples 454, 476, 477, and 444, of the WO 00/62778 application, respectively.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Fei Huang

Fei Huang, Ph.D.

Aug 22, 2006

Date

Enclosures: Exhibits A, B, C, D, E, F, G, H, I, J, K, and L.